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Reconstitution of β_1-adrenoceptor-dependent adenylate cyclase from purified components

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In continuation of our efforts to reconstitute from purified components into lipid vesicles the signal transmission chain from β_1-adrenoceptors to adenylate cyclase, we now report on the total reconstitution of the hormone-dependent adenylate cyclase. In these reconstitution experiments we have employed the purified adenylate cyclase (C) from bovine brain and rabbit heart, the stimulatory GTP-binding protein (G_s) purified from turkey erythrocytes and rabbit liver and the β_1-adrenoceptor (R) from turkey erythrocytes. Several detergents were compared with respect to their suitability to allow reconstitution of subunits into phospholipid vesicles. While octyl–polyoxyethylene (octyl–POE) was almost as potent as lauroyl–sucrose for preparation of vesicles containing G_s.C, the latter detergent was clearly superior for vesicles enabling productive R.G_s and R.G_s.C coupling. The catalytic subunit from either bovine brain or rabbit heart was equally efficient in reconstitution. However, G_s from turkey erythrocytes and rabbit liver revealed significant differences in RG_s and RG_s.C containing vesicles. While isoproterenol-induced activation of G_s by GTPγS was first order in both instances, k_{on} with turkey G_s was 0.12 min^{-1}, whereas k_{on} with rabbit liver G_s was 0.6 min^{-1}. Moreover, GTPγS activation of erythrocyte G_s was significantly more dependent on the presence of hormone than that of liver G_s, confirming observations made on the native membrane-bound system. Compared with stimulation by isoproterenol (GTPγS) (4-fold), stimulation by isoproterenol/GTP was modest (1.3- to 1.6-fold). The difference between activation by GTP and GTPγS, and the lag in onset of cAMP production with GTPγS but not with GTP, resembles the situation in the native membrane-bound system. These findings provide further evidence that the reconstituted system accurately reflects the signal transmission sequence of native membranes.

Key words: adenylate cyclase/β_1-adrenoceptor/G_s/GTP/GTPγS/lipid vesicles/reconstitution

Introduction

Hormonally regulated adenylate cyclase is a transmembrane signalling system responsible for the control of intracellular levels of cAMP. Stimulatory hormones activate the catalytic unit of adenylate cyclase (C) by interaction with the stimulatory GTP regulatory protein G_s (or N_s) associated with the catalyst (Helmreich and Pfeuffer, 1985; Levitzki, 1985), whereas inhibitory hormones interact with another GTP regulatory protein, G_i, which mediates adenylate cyclase inhibition. So far, the only available purified stimulatory receptor is the β-adrenoceptor (R) which has been purified from a number of sources (Shorr *et al.*, 1982; Homcy *et al.*, 1983; Cubero and Malbon, 1984; Benovic *et al.*, 1984; Hekman *et al.*, 1984). G_s has also been purified from a number of tissues (Sternweis *et al.*, 1981; Hanski *et al.*, 1981; Pfeuffer *et al.*, 1983). The availability of purified components made it possible to study the activation of G_s by β-adrenoceptors in reconstituted vesicle preparations (Brandt *et al.*, 1983; Cerione *et al.*, 1984; Asano and Ross, 1984; Asano *et al.*, 1984; Hekman *et al.*, 1984). These results confirmed earlier data on membranes and allowed more detailed experiments on the mechanism of R to G_s coupling (for review, see Levitzki, 1985). However, the total reconstitution of hormone-sensitive adenylate cyclase was dependent on the purification of the catalytic unit. This has recently been achieved in the isolation of pure catalyst from two sources: rabbit heart (Pfeuffer *et al.*, 1983, 1985b) and bovine brain (Pfeuffer *et al.*, 1985a). Thus, the availability of pure β_1-adrenoceptors from turkey erythrocytes, pure G_s from turkey erythrocytes and rabbit liver, and pure C from bovine brain made it possible to attempt total reconstitution, using three separate functional units in their pure form.

A recent report by May *et al.* (1985) describes the functional reconstitution of turkey erythrocyte β_1-adrenoceptor with pure rabbit liver G_s and bovine brain C. In this study we report in detail on the successful reconstitution of hormonally activated adenylate cyclase from the components R, G_s and C. In addition, we show that G_s from two different sources, and C from two different mammalian tissues, can be functionally reconstituted.

Results

Purity of the components

Purified turkey erythrocyte R was prepared as described (Hekman *et al.*, 1984). G_s protein from turkey erythrocytes and from rabbit liver were purified as described (Sternweis *et al.*, 1981; Hanski *et al.*, 1981). Purified catalytic unit of adenylate cyclase from rabbit heart and from bovine brain were utilized throughout this study. The purity of the components used can be assessed from Figure 1.

Choice of detergent for reconstitution

We have previously noted (Hekman *et al.*, 1984) that the detergent lauroyl-sucrose is very efficient in bringing about effective R to G_s coupling. We have also noted earlier (Arad *et al.*, 1984) that G_s to C coupling is efficient in octyl–polyoxyethylene (POE). Therefore, we have compared the two detergents as well as the widely used octyl–glucoside, and tested them for suitability in coupling of G_s to C and for coupling of the β_1-adrenoceptor to G_s. Figure 2 shows that, although octyl–POE allows G_s to C coupling nearly as well as lauroyl–sucrose, when measured with $AlCl_3$/$Mg(Ac)_2$/NaF (AMF), and somewhat better when forskolin stimulation is monitored, lauroyl–sucrose was superior

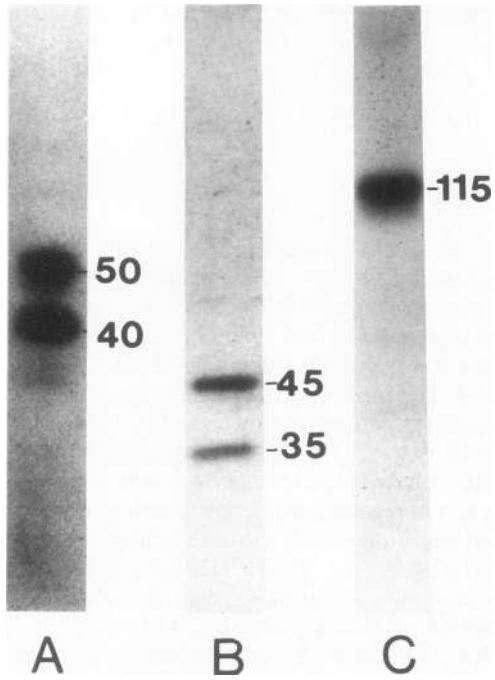

Fig. 1. SDS–polyacrylamide gels of the three protein components of the adenylate cyclase complex used for reconstitution. **Lane A:** β_1-adrenoceptor from turkey erythrocyte membranes (40 ng) ^{125}I-iodinated. The receptor subunits are 40 kd and 50 kd. **Lane B:** G_s from turkey erythrocyte membranes (2.3 μg) stained with Coomassie blue. The α_s (45 kd) and the β (35 kd) subunits are clearly seen. **Lane C:** catalytic subunit (C) purified from bovine brain cortical membranes (75 ng) ^{125}I-iodinated. **Lanes A** and **B**, 11% and 9% polyacrylamide gels; **lane C**, 4–14% linear-gradient polyacrylamide gel according to Laemmli (1970). Numbers refer to mol. wts in kd. **Lanes A** and **C**, autoradiograms.

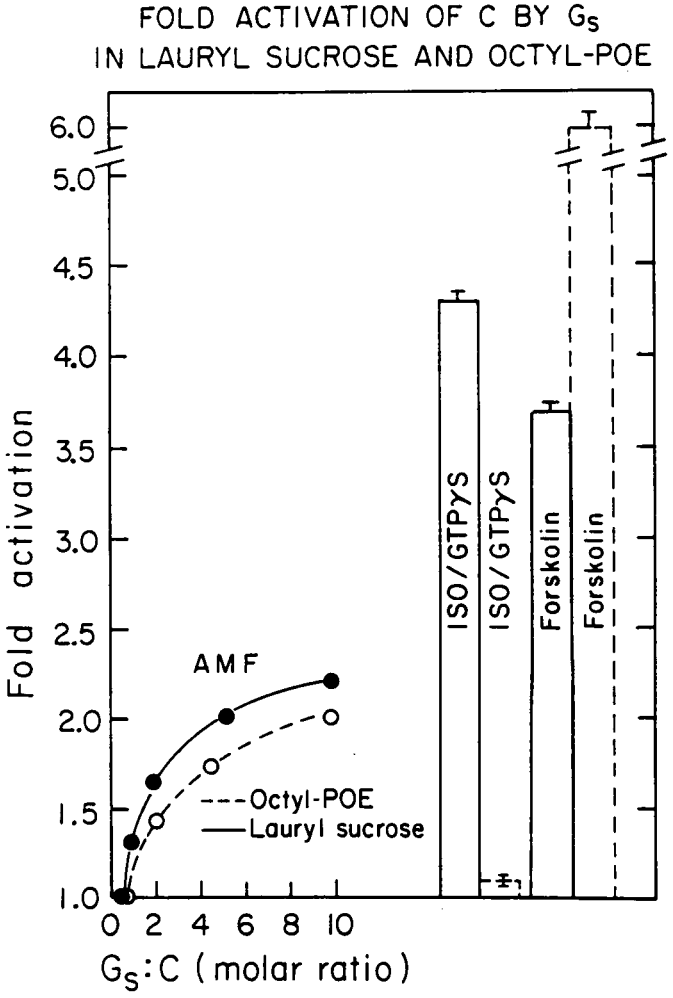

Fig. 2. Reconstitution of C with G_s in vesicles formed in the presence of lauroyl–sucrose or octyl–POE. Bovine brain C (150 fmol) was reconstituted with 75–1500 fmol of deactivated turkey erythrocyte G_s, as described in Materials and methods, using the G-50 Sephadex reconstitution procedure. Adenylate cyclase activity was measured in the presence of either 10^{-6} M GTPγS or AMF.

to both octyl–POE and octyl–glucoside for R to G_s coupling (Figure 3). In the R/G_s/C reconstitution, the difference between lauroyl–sucrose and octyl–POE is striking (Figure 2, right panel). Stimulation of the reconstituted system with 1-isoproterenol and GTPγS yields 4.3 ± 0.2-fold stimulation of C over basal. With octyl–POE only 1.1 ± 0.05-fold stimulation is obtained. The absolute level of stimulation by 1-isoproterenol and GTPγS is ~70–90% of the maximal attainable adenylate cyclase stimulation obtained with 20 mM Mg^{2+} in the presence of Lubrol–PX. These findings convinced us to choose lauroyl–sucrose for further reconstitution experiments.

Coupling of the β-adrenoceptor with G_s from two different sources

Pure β_1-adrenoceptors catalyze the GTPγS-dependent activation of pure G_s from either turkey erythrocytes or rabbit liver. In these experiments, activated G_s was measured by following cAMP production on activation of a crude adenylate cyclase preparation from myocardial membranes (cf. Hekman *et al.*, 1984). In both cases, the kinetics are first order but the rate constant for isoproterenol activation of rabbit liver G_s is five times greater than that for turkey erythrocyte G_s (Figure 4). Receptor-independent activation of rabbit liver G_s is also 11 times faster than that of turkey erythrocyte G_s. These differences may reflect the difference in structure of the two G_s proteins (see Discussion).

Reconstitution of a β-receptor-sensitive cyclase with G_s from two sources

The difference between rabbit liver G_s and turkey erythrocyte G_s is also reflected in reconstitution experiments where purified bovine brain C was co-reconstituted with β_1-adrenoceptor and either rabbit liver G_s or turkey erythrocyte G_s. In both cases, a characteristic 'lag' (reflecting k_{on}) in the production of cAMP is observed. Moreover, rabbit liver G_s is readily activated with GTPγS to a considerable extent in the presence of the β-blocker propranolol, while the rate and extent of hormone-independent activation of turkey erythrocyte G_s under identical conditions is much smaller (Figure 5). This finding confirms earlier data reported on the behavior of the adenylate cyclase system in turkey and liver membranes, which indicated that such differences are determined mainly by the intrinsic nature of the G-components (Londos *et al.*, 1974; Kaslow *et al.*, 1979). Maximal isoproterenol stimulation of C via G_s is observed at GTPγS concentrations of 1–10 μM in the reconstitution mixture, although significant stimulations are also observed at lower concentrations of GTPγS (data not shown).

Reconstitution of β-receptor sensitive cyclase with C from two sources

Bovine brain C and rabbit heart C were found to be about equipotent in reconstitution experiments using turkey erythrocyte G_s

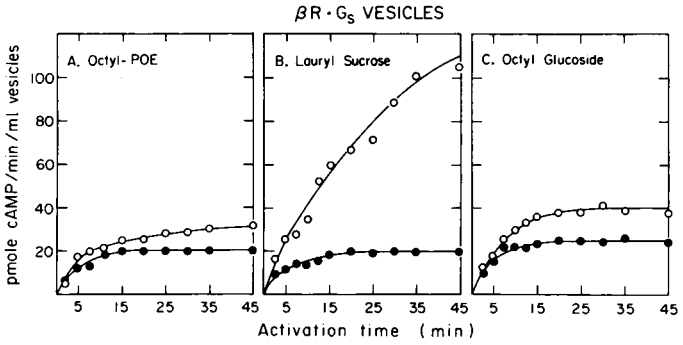

Fig. 3. Efficiency of lauroyl−sucrose, octyl−POE and octyl−glucoside in formation of vesicles containing R and G_s. Vesicles were prepared from 2.7 pmol of deactivated turkey erythrocyte G_s, and 2.5 pmol of R.G_s was activated in the presence of 100 μM d,l-propranolol + 40 nM GTPγS (●) or 10 μM l-isoproterenol + 40 nM GTPγS (○).

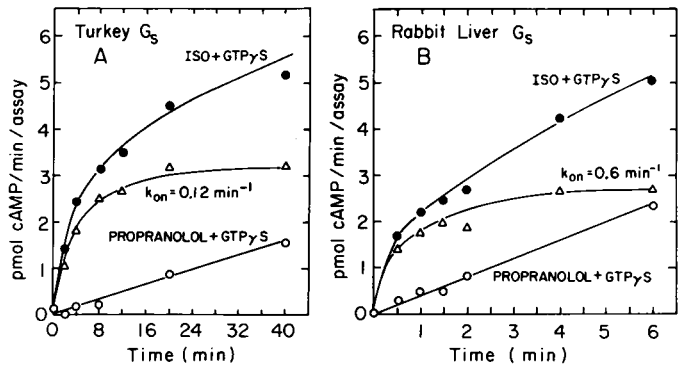

Fig. 4. Kinetics of activation of rabbit liver G_s and of turkey erythrocyte G_s by turkey erythrocyte β_1-adrenoceptor. Purified β_1-adrenoceptor (6 pmol) was mixed with 21 pmol of G_s from either source in 70 μl 0.6 mM Lubrol−PX, 15 μl lauroyl−sucrose 6% (final concentration 0.3%), 12 μl asolectin (20 mg/ml, final concentration 0.6 mg/ml), and 15 μl BSA (20 mg/ml, final concentration 1 mg/ml) in a final volume of 300 μl. This mixture was then reconstituted by the Extracti-Gel D method, described in Materials and methods. The reconstituted material contained 3.1 pmol R/ml for the liver G_s system and 3.3 mol/ml for the turkey G_s system. Time-dependent activation of G_s in the presence of 1 μM GTPγS and 10 μM l-isoproterenol (●), or 1 μM GTPγS and 10 μM d,l-propranolol (○) was measured in Hekman *et al.* (1984). (△) is the calculated difference between (●) and (○).

and R (Figure 6). However, the greater instability of the myocardial enzyme makes its usage disadvantageous.

Comparison of GTPγS and GTP

The natural activator of G_s is GTP; hence, it is of crucial importance to examine the ability of GTP to activate G_s in the presence of (−)isoproterenol in a reconstituted system which must include C in order to catch the transiently formed G_s.GTP complex. Whereas stimulation in the presence of GTPγS/isoproterenol shows the typical lag (Figures 5 and 6), stimulation by GTP/isoproterenol displays no lag (Figure 7), similar to the situation in the native membrane (Tolkovsky and Levitzki, 1978). In addition, GTP/isoproterenol stimulates much less (12%) effectively than GTPγS/isoproterenol, which is characteristic of the turkey erythrocyte adenylate cyclase system (Sevilla *et al.*, 1976).

The role of phospholipid composition

A combination of phosphatidyl-ethanolamine (PE) and phosphatidylserine (PS) (3:2, w/w) yields similar results to soybean lipids in reconstitution experiments of R with G_s and C, when the system is activated by GTPγS. When GTP is the activating nucleotide, however, a somewhat higher signal (isoproterenol + GTP versus propranolol + GTP) is observed when the PE/PS mixture is compared with soybean lipids (Figure 8).

Discussion

Universality of the system

The complete purification of the catalytic unit of adenylate cyclase made it possible to attempt the total reconstitution of receptor-dependent adenylate cyclase from its pure separate components. In the present study, we have demonstrated that turkey erythrocyte β_1-adrenoceptor can interact efficiently with both turkey erythrocyte G_s and rabbit liver G_s (Figures 4 and 5). These results show that the three protein components R, G_s and C units, are sufficient to generate hormone-dependent adenylate cyclase activation. This result does not exclude the importance of other components such as G_i or free β,γ-subunits, as suggested by Cerione *et al.* (1985), to 'fine-tune' the system. Our results cannot be directly compared with those of Cerione *et al.* (1985), as the latter have used a crude preparation of adenylate cyclase for their reconstitution experiments.

The rate constant of R catalyzed G_s activation is k_{on} = 0.6

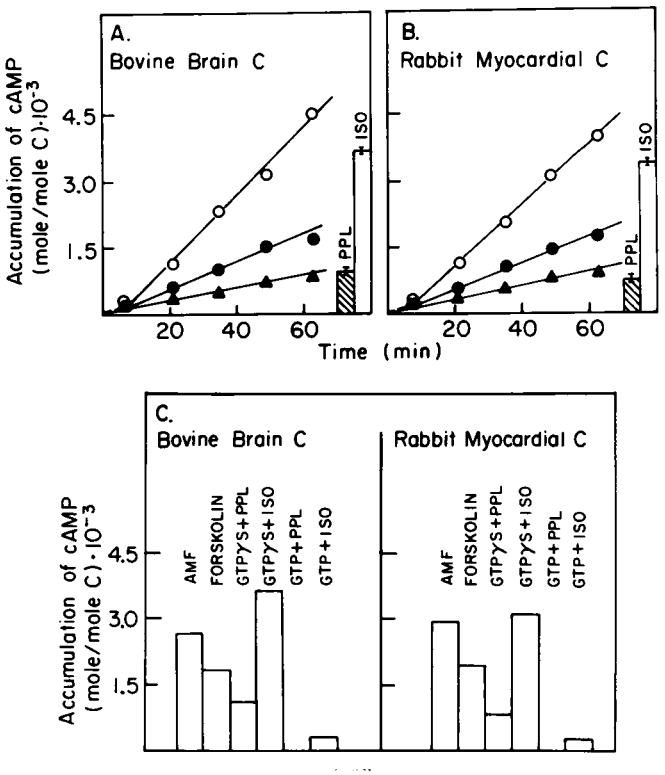

Fig. 5. Reconstitution of the R·G_s·C complex using G_s from two sources. R (4.5 pmol) was reconstituted with 14 pmol of bovine brain C and 58 pmol of either turkey erythrocyte G_s or rabbit liver G_s, as described in Materials and methods. Adenylate cyclase activity in vesicles was measured in the presence of 10 μM d,l-propranolol + 1.0 μM GTPγS (●), 100 μM l-isoproterenol + 1.0 μM GTPγS (○) or no addition (▲). **(A)** Turkey erythrocyte G_s; **(B)** rabbit liver G_s. **(C)** histogram of adenylate cyclase activity minus basal activity (turkey erythrocyte = 1.5 × 10^3, rabbit liver = 1.8 × 10^3) at 60 min.

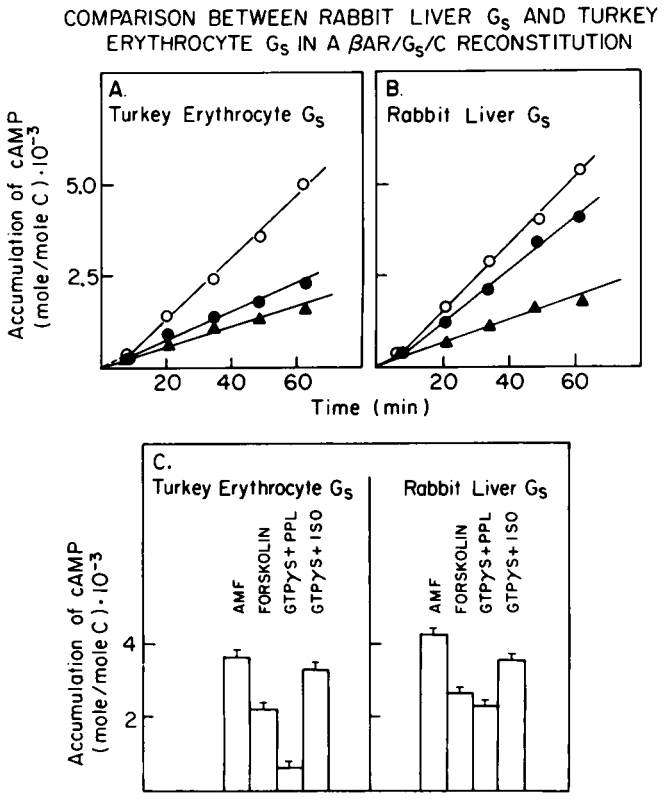

Fig. 6. Reconstitution of the R.G_s.C complex using C from two sources. Reconstitution was performed with 0.6 pmol of C, 11.4 pmol of turkey erythrocyte G_s and 6.5 pmol of pure β_1-adrenoceptor, with 0.6 mg/ml asolectin in a final volume of 315 μl, as described in Materials and methods. Adenylate cyclase activity in the presence of 10 μM d,l-propranolol − 1.0 μM GTPγS (●), 10 μM l-isoproterenol + 1.0 μM GTPγS (○), or no addition (▲) is shown. **(A)** Bovine brain C; **(B)** rabbit myocardial C; **(C)** histogram of adenylate cyclase activity minus basal activity (bovine C = 0.9 × 10³, rabbit myocardial C = 0.93 × 10³) at 60 min.

min^{-1} in the case of rabbit liver G_s as compared with turkey erythrocyte G_s (k_{on} = 0.12 min^{-1}) under identical conditions (Figure 4). Rabbit liver G_s has two forms of α_s, namely, 52-kd and 45-kd proteins (Sternweis *et al.*, 1981), while turkey erythrocyte possesses only the 45-kd subunit (Hanski *et al.*, 1981). It has already been shown that the 52-kd α_s-subunit is more readily activated by guanyl nucleotides than the 45-kd subunit (Hanski *et al.*, 1981), and that rabbit liver G_s is more readily activated by guanyl nucleotides than turkey erythrocyte G_s. From the R/G_s/C reconstitution experiments (Figure 5), it is indeed apparent that activation of turkey erythrocyte G_s is more receptor-dependent than that of rabbit liver G_s. This resembles the situation observed with the native membrane system (Londos *et al.*, 1974; Kaslow *et al.*, 1979).

Bovine brain C and rabbit heart C were found to be about equally effective in reconstitution experiments with turkey erythrocyte G_s and turkey erythrocyte β_1-adrenoceptor (Figure 6). Thus, these cross-experiments employing G_s units and C units from different tissues and species to obtain (−)isoproterenol-responsive adenylate cyclase activity demonstrate that the G_s to C and R/G_s interaction domains are conserved across species in the course of evolution.

GTPγS versus GTP as activators

In the turkey G_s system, the relative potencies of GTP and GTPγS in the presence of hormone are very different. For exam-

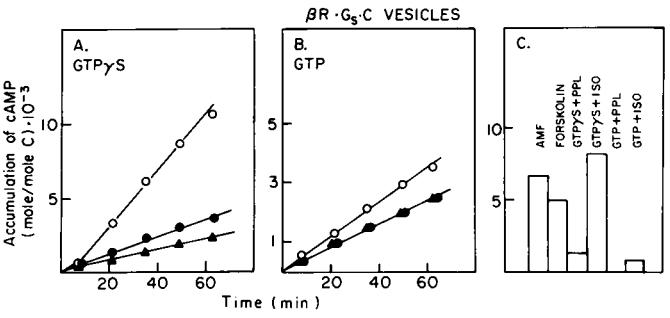

Fig. 7. The hormonal activation of R·G_s·C vesicles by GTPγS versus GTP. Vesicles were prepared from 14 pmol of pure β_1-adrenoceptor, 32 pmol of turkey erythrocyte G_s and 8 pmol of bovine brain C, with 0.6 mg/ml asolectin, in a final volume of 315 μl, as described in Materials and methods. The phospholipid to protein ratio was (w/w) 220:1 for R, 85:1 for G_s and 212:1 for C. (●), 10 μM d,l-propranolol; (○), 100 μM l-isoproterenol; (▲), no additions. **(A)** Activation with GTPγS, 1 μM; **(B)** activation with GTP, 1 μM; **(C)** histogram of adenylate cyclase activity minus basal activity at 60 min (basal = 2.5 × 10³).

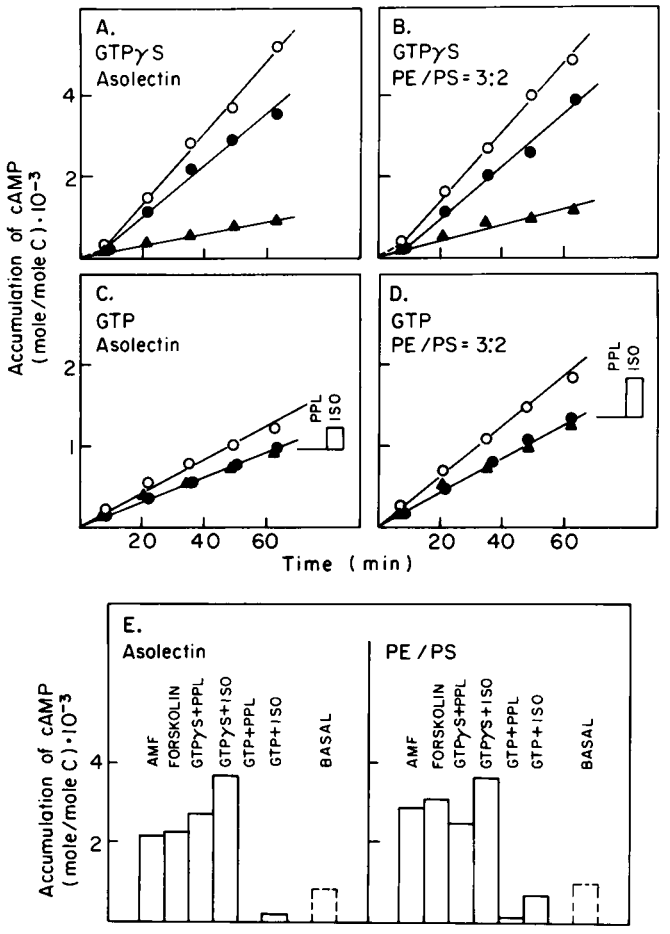

Fig. 8. The relative potencies of GTPγS and GTP in stimulating adenylate cyclase as a function of lipid composition of the vesicles. Vesicles were prepared from 16.7 pmol of R, 28.4 pmol of rabbit liver G_s and 12 pmol of bovine brain C, with 1.1 mg/ml asolectin **(A** and **C)** or a mixture of PE/PS (0.66/0.44 mg/ml) **(B** and **D)**. The total reconstitution volume was 374 μl in each case. The phospholipid to protein ratio was (w/w) 325:1 for R, 85:1 for G_s, and 137:1 for C. Adenylate cyclase activity in the presence of 10 μM d,l-propranolol (●), 100 μM l-isoproterenol (○), or no addition (▲) is shown. **A** and **B**, activation with GTPγS (1 μM) in asolectin **(A)** and PE/PS vesicles **(B)**. **C** and **D**, activation with GTP (1 μM) in asolectin **(C)** and PE/PS vesicles **(D)**.
E, histogram of adenylate cyclase activity minus basal activity at 60 min (**A,C** = 0.9 × 10³; **B,D** = 1.1 × 10³).

ple, GTP produced only 1.3- to 1.6-fold (Figure 7) stimulation compared with ~4-fold stimulation (Figures 2, 5 and 7) with GTPγS. In the rabbit liver G_s system, the difference is less extreme, as found in the rabbit liver membrane (Londos *et al.*, 1974). This is in good agreement with the data of May *et al.* (1985) who likewise employed rabbit liver G_s in their reconstitution experiments, observing a modest enhancement (20–30%) by isoproterenol of GTPγS-activated adenylate cyclase. The GTP/GTPγS potency ratios correlate well to findings in native systems (Kaslow *et al.*, 1979).

The extent of stimulation of pure C by G_s and R in the presence of GTP was sensitive to the phospholipid composition of the vesicles. In the rabbit liver G_s system, when the components were reconstituted into vesicles made of PE/PS (3:2, w/w), the GTP/iso stimulation is 1.7 ± 0.1-fold as compared with 1.37 ± 0.1-fold when soybean lipids (asolectin) are used under identical reconstitution conditions. With GTPγS we obtained the same (−)isoproterenol activations in both types of vesicles (Figure 8). May *et al.* (1985), who report a 2.3-fold activation by GTP/iso using identical components (turkey erythrocyte R, rabbit liver G_s and bovine brain C), also utilized a phospholipid mixture composed of PE and PS at a ratio of 3:2 (w/w). Our results, like those of May *et al.* (1985), indicate the need for further quantitative studies of the factors involved, including the role of phospholipids.

Kinetic features of the reconstituted system

β_1-Adrenoceptor-catalyzed activation of G_s is first order for both rabbit liver G_s and turkey erythrocyte G_s (Figure 4), confirming previous results (Hekman *et al.*, 1984). Also, when a non-hydrolyzable analog of GTP instead of GTP is used in the reconstituted R.G_s.C system, the 'lag' characteristic for the activation of adenylate cyclase by β-receptors is apparent (Figures 5 and 6). No lag is observed with GTP (Figures 7 and 8). Thus, the kinetics resemble the behavior of β_1-adrenoceptor-dependent adenylate cyclase in native systems (Sevilla *et al.*, 1976; Tolkovsky and Levitzki, 1978). The ability of the reconstituted system to reproduce the basic kinetic characteristics of hormone-dependent adenylate cyclase activation gives confidence in the fidelity of the experimental approach.

The ratio of phospholipid to protein component used in the reconstitution experiments is in the range of 200–700 (w/w). This ratio is similar to the phospholipid to protein ratios used in reconstitution experiments reported by others (Cerione *et al.*, 1985; May *et al.*, 1985). If the incorporation of the protein components into the phospholipid vesicles were solely governed by statistical rules, no vesicles with all three components would have been obtained (Levitzki, 1985). The fact that the three components couple effectively in the reconstituted system, despite the unfavorable phospholipid to protein ratio, suggests that the components associate with one another in the detergent-soluble state prior to and during vesiculation, which enables their insertion into the same vesicle.

Choice of detergent and reconstitution protocol

We have previously noted that lauroyl–sucrose, in contrast to deoxycholate and cholate, does not denature the components R, G_s and C (Feder *et al.*, 1984; Hekman *et al.*, 1984). We have used, therefore, exclusively this detergent in our present reconstitution experiments. It is also apparent that the efficiency of coupling between the components when reconstituted from lauroyl–sucrose is high.

Conclusions

The recently developed reconstitution assays using pure components (this report and May *et al.*, 1985) are a hopeful beginning towards the aim of providing a tool for the study of kinetics, stoichiometry and regulation of the signal transmission chain from β-receptor to adenylate cyclase. The response of the reconstituted system to the natural effector GTP clearly shows that further efforts are required to optimize the system. However, the considerable efforts required to reach this goal are worthwhile, because a reconstitution system made up of homogeneous components is a prerequisite for a study of the molecular interactions between complementary domains of the coupling partners.

Materials and methods

Materials

Chemicals and biochemicals were obtained from the same sources as described before (Pfeuffer *et al.*, 1985; Hekman *et al.*, 1984), unless otherwise specified. Lauroyl–sucrose was obtained from Mitsubishi Co. (Japan) and recrystallized from cold (−20°C) acetone overnight. Asolectin, PS, PE and octyl–glucoside were purchased from Sigma. Extractigel-D was from Pierce. [^{14}C]phosphatidylcholine was from Amersham and [^{3}H]Lubrol–PX was synthesized according to Gaylor and Delwiche (1969). Octyl–POE was a generous gift of Dr J.Rosenbusch, Basle, and forskolin was kindly supplied by Drs Schöne and Schorr, Hoechst AG, Frankfurt.

Membrane preparations

Purified turkey erythrocyte membranes were prepared according to Puchwein *et al.* (1974). Bovine brain membranes were prepared according to Pfeuffer *et al.* (1985a), and rabbit heart membranes as described earlier (Pfeuffer and Metzger, 1982). Protein concentration was determined according to Schultz *et al.* (1978).

Adenylate cyclase assay

Enzyme activity was measured in a final volume of 75 μl, containing 20 mM MOPS, pH 8.0, 45 mM NaCl, 0.1 mM ATP, 5 mM theophylline, 0.2 mg/ml creatine kinase, 10 mM creatine phosphate, 2.4 mM $MgCl_2$ and, 2–4 μCi/[α-^{32}P]ATP per assay. In some experiments the ATP regenerating system was omitted. When activation by F^- was assayed, 10 mM NaF, 20 μM $AlCl_3$ and 6 mM $MgCl_2$ (AMF) were present. Forskolin-stimulated activity was measured in the presence of 100 μM forskolin. The assay was carried out at 30°C, and the [^{32}P]cAMP was measured according to Salomon *et al.* (1974).

G_s purification

The purification of G_s from turkey erythrocytes and rabbit liver membranes was conducted according to Hanski *et al.* (1981) and Sternweis *et al.* (1981), respectively. Deactivation of G_s was performed by slow application (1–4 h) of 4–15 ml of G_s onto a 200–400 μl hydroxyapatite column (Bio-Rad HTP, DNA grade), pre-equilibrated with 25 mM MOPS, 0.1 mM EDTA, 100 mM NaCl and 1 mM dithiothreitol (DTT), pH 7.5, containing 1 mM (0.06%) Lubrol–PX. The column was then washed with 10 ml of the same buffer, followed by a 15 ml gradient of 1–20 mM potassium phosphate, pH 7.5, 1 mM Lubrol–PX. G_s was eluted subsequently with 300–750 μl of 400 mM potassium phosphate, pH 7.5, 1 mM Lubrol–PX. The eluted material was de-salted by centrifugation (2 min) of 200 μl aliquots through a syringe containing 2 ml of dehydrated Biogel P-4 (Bio-Rad) at 1000 *g*. The gel had been previously washed with 20 mM MOPS buffer, pH 7.8, 0.1 mM EDTA, 100 mM NaCl, 1 mM DTT, 1 mM Lubrol–PX, 1 mg/ml of bovine serum albumin (BSA) and then with the same solution without BSA. All operations were carried out at 4°C. The deactivated G_s was stable for at least 10 days at 4°C when stored in 20% glycerol. G_s from both sources was usually >90% pure and did not include G_i or free β,γ-subunits.

Purification of the catalyst

The catalytic subunit of adenylate cyclase was purified from either bovine brain (Pfeuffer *et al.*, 1985a) or rabbit myocardial membranes (Pfeuffer *et al.*, 1985b). The catalyst used in this study was >95% pure (bovine brain cortex) and 20–30% pure (rabbit myocard) as judged by SDS gel electrophoresis. Forskolin was removed from the catalyst on a hydroxyapatite column as described (Pfeuffer *et al.*, 1985a). Briefly, the purified enzyme was applied onto a 100 μl hydroxyapatite column, pre-equilibrated with 10 mM MOPS, 1 mM $Mg(Ac)_2$, 1 mM EDTA, 100 mM NaCl and 1 mM Tween 60, pH 7.4. The column was then washed with 1 ml of the same buffer, followed by elution with 300 μl of 400 mM potassium phosphate, pH 7.4/1 mM Lubrol–PX. De-salting was carried out with Biogel P-4 (Bio-Rad), equilibrated with 10 mM MOPS, pH 7.4, 100 mM NaCl,

1 mM Lubrol−PX, as described above for the de-salting of G_s. The overall recovery in this step is 50%. The forskolin-free bovine brain catalyst is stable in 20% glycerol for at least 10 days, while the myocardial catalyst loses 50% of its activity within 5 days under identical conditions.

Purification of β-adrenoceptor

The method of Hekman *et al.* (1984) was used, modified as follows: 1 g of washed turkey erythrocyte membranes (1.3 ± 0.1 pmol R/mg) were solubilized in 250 ml 0.7% lauroyl−sucrose in 20 mM Hepes buffer, pH 7.5, 90 mM NaCl, by stirring at 4°C for 40 min, followed by centrifugation at 40 000 *g* for 1 h. The solubilized material, 200−250 ml, 3.5−4 pmol [^{3}H]di-hydroalprenolol (DHA)-binding sites/ml (1.8− 2.2 mg protein/ml), was stirred with 10 ml Affigel 10-alprenololamine, pre-equilibrated with 0.3% lauroyl−sucrose in the above buffer for 3 h at room temperature or overnight at 4°C. The loaded gel was applied to a column (2 cm diameter) and washed for 24 h at 4°C with 400 ml of 20 mM Hepes buffer, pH 7.5, 0.5 M NaCl (or 1.0 M NaCl for Affigel 15-alprenololamine), in the presence of protease inhibitors and 0.3% lauroyl−sucrose (Hekman *et al.*, 1984). About 70−80% of the receptor added to the gel was retained. Receptor was then eluted with 40 ml of a solution containing 5 mM isoproterenol, 0.1 M ascorbic acid, 20 mM Hepes buffer, pH 7.6, 250 mM NaCl, 2 μM phenyl-methylsulphonyl fluoride (PMSF), 0.05 μg/ml of soybean trypsin inhibitor and 0.3% lauroyl−sucrose. Elution was performed at room temperature at a rate of 5 ml per hour. The first 10 ml were discarded and the following 20 ml of the effluent were directly passed over a hydroxyapatite (Bio-Rad, DNA grade) column (150−200 ml), pre-washed with 0.3% lauroyl−sucrose in 20 mM Hepes, pH 7.5, at 4°C. The hydroxyapatite column was then washed with 20 ml of 20 mM Hepes buffer, pH 7.5, 90 mM NaCl and, subsequently, with 10 mM phosphate buffer, pH 7.6, and 0.3% lauroyl−sucrose. Bound R was then eluted with four portions of 100 μl of 400 mM potassium phosphate, pH 7.5, and 0.3% lauroyl−sucrose. For removal of phosphate, the eluate was transferred to a Centricon (Amicon) filter device and, following addition of 1.5 ml of 20 mM Hepes buffer, pH 7.6, concentrated to a minimal volume. The procedure was repeated four times. This protocol yielded ~300 μl of concentrated pure receptor (~600 pmol/ml) with ~20% overall yield. SDS polyacrylamide gels (Figure 1) show two polypeptides with mol. wts of 40 and 50 kd at a ratio of ~4:1 (Hekman *et al.*, 1984).

G_s to C reconstitution

Forskolin-free bovine brain adenylate cyclase 0.2 pmol and 0.1−2.0 pmol G_s were reconstituted in a final volume of 400−800 μl containing 0.2−0.6 mg/ml of BSA, 0.45% octyl−POE or 0.2% lauroyl−sucrose, 0.8 mg/ml asolectin in 20 mM MOPS, pH 8.0, 45 mM NaCl. The reconstitution mixture was held on ice for 10 min and then applied to a cold (4°C) 10 ml Sephadex G-50 (fine) column, pre-equilibrated with 10 mM MOPS, pH 8.0, 0.5 mM DTT, 0.4 mM $MgCl_2$, 0.2 mM EDTA and 5% glycerol. Incorporation of G_s and C was usually ~50% in vesicles. Aliquots (30 μl) of fractions were assayed for basal, AMF- and forskolin-stimulated activities.

R/G_s reconstitution

The G_s:R ratio in the reconstitution mixture was 0.5−2:1 (2.5 pmol R). Following addition of 0.45% octyl−POE or 0.3% lauroyl−sucrose or 0.85% octylglucoside, vesiculation was achieved applying the same conditions as described for G_s to C coupling. Activation of G_s was conducted at 30°C with 40 nM GTPγS in the presence of either 100 μM l-isoproterenol or 10 μM d,l-propranolol and 0.28 mM $Mg(Ac)_2$. The reactivation was stopped by aliquoting 30 μl into 5 μl of 0.6 mM propranolol and GTP, each, on ice. Active G_s was determined as described before (Hekman *et al.*, 1984).

R/G_s/C reconstitution

These experiments employ the three components at high concentrations (200−350 pmol/ml) with molar ratios of 1−6 G_s to R, and 3−15 G_s to C. The free Mg^{2+} was 0.5 mM, and asolectin was usually 0.6 mg/ml. The reconstitution mixture was kept on ice for 5 min, and vesiculation was achieved by either the Sephadex G-50 method or by the Extracti-Gel D procedure (see below). After vesiculation, the reconstituted material was assayed for adenylate cyclase activity at 30°C by adding a vesicle sample to the reaction mixture, both pre-incubated at 30°C.

Extracti-Gel D method

The Extracti-Gel method for reconstitution yields similar results as the Sephadex G-50 method, but has the advantage of requiring less time, and gives more uniform results. Extracti-Gel D is first washed with 9 volumes of 10 mM MOPS, pH 8.0, 0.5 mM DTT, 0.4 mM $Mg(Ac)_2$, 0.2 mM EDTA, 5% glycerol, 100 mM NaCl and 2 mg/ml BSA. Before reconstitution, the gel is washed with the same buffer but without BSA. Subsequently, the reconstitution mixture (1.5 volumes) is added to the gel in a small syringe and agitated for 30−40 min at 4°C. Formed vesicles are eluted by positive pressure. [^{3}H]Lubrol−PX and [^{14}C]phosphatidylcholine were occasionally added to monitor detergent removal and phospholipid incorporation. Lubrol−PX is removed by >98%, while phospholipid incorporation is typically 60%.

Originally we used a reconstitution protocol similar to the one used by Ross and his colleagues (Pedersen and Ross, 1982), where the reconstitution mixture is passed through a Sephadex G-50 column for vesiculation (Hekman *et al.*, 1984). We have recently found that reconstitution on Extracti-Gel D (Pierce) yields similar results, but the latter method requires less time, is more efficient in the removal of detergent from small reconstitution volumes and provides more uniform results. We have therefore adopted this method and have used it, together with the former procedure, for many experiments.

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